Fenitrothion toxicity in *Triatoma infestans* synergized by quercetin or thymol blue

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Abstract: Quercetin and thymol blue were shown to synergize the toxicity of fenitrothion to *Triatoma infestans* with synergistic ratios of 1.89 and 2.65 respectively. These synergistic ratios were statistically significant at $P < 0.05$.

Both compounds inhibited glutathione *S*-transferases (GST) *in vitro*, with pI_{50} values of 6.1 and 5.1 respectively. Quercetin or thymol blue caused in-vivo GST inhibition without affecting non-specific esterase (NSE) or acetylcholinesterase (AChE) activity.

Incubation of [^{14}C]fenitrothion with *T. infestans* or rabbit liver GST produced desmethyl-fenitrothion as the major metabolite, which was specifically diminished in the presence of 0.3 mM quercetin.

[^{14}C]Fenitrothion toxicokinetics study showed a significant decrease ($P < 0.05$) in radioactivity due to polar metabolites when insects were pre-treated with quercetin. These facts suggest that both assayed chemicals may be active in synergizing fenitrothion toxicity due to their ability to prevent the detoxification of organophosphorus insecticides by GSH conjugation.

Keywords: *Triatoma infestans;* fenitrothion synergism; glutathione *S*-transferase (GST) inhitition; quercetin or thymol blue as inhibitors of GST

1 INTRODUCTION

Chagas disease or American Trypanosomiasis is found on the American continent in the area between 42°N latitude and 45°S latitude. It is caused by a flagellate protozoan parasite, *Trypanosoma cruzi* (Chagas), which is transmitted to humans in the faeces of blood-sucking triatomine reduviid bugs, such as *Triatoma infestans* (Klug), which is the main vector in Argentina.

Because infected humans remain infective throughout their lives and there is neither cure nor immunological protection, chemical control of the vectors appears to be the best way to reduce the impact of the disease.[1,2] The chemical control has been based almost entirely on spraying of houses and adjacent structures in rural areas with chlorinated hydrocarbon, organophosphorus (OP) or pyrethroid insecticides. Among the OP insecticides fenitrothion has been the most effective for controlling Chagas disease vectors,[3] but is much less effective than deltamethrin (pyrethroid).[4,5]

Most insecticides are initially metabolized in insects by oxidation, hydrolysis or conjugation. Metabolism by these mechanisms could result in activation or detoxification of the insecticide.[6] Thus, selectivity is frequently related to the rate at which these reactions occur in various insects or various living organisms.[7]

The glutathione *S*-transferases (GST) (E.C.2.5.1.18) represent a group of multifunctional proteins performing several roles in detoxification.[8,9] All detoxification functions of the transferases are the result of a single feature of these proteins: their capacity to bind many different and varying chemicals that are hydrophobic. Basically, GST can have two functions: that of sequestration of non-substrate ligands and that of catalytic activity, both important in detoxification. GST of non-vertebrate organisms have not received the detailed scrutiny afforded to those of mammals.[10,11] Nevertheless, they are of interest because of the involvement of the enzymes in protecting the organism from toxic foreign compounds in the environment.[12,13] Insect GST metabolize various electrophilic xenobiotics, including halogenated compounds, nitrocompounds, *alpha*- or *beta*-unsaturated compounds, isothiocyanates, organothiocyanates, organophosphorus compounds and oxides. At least 35 chemicals have served as substrates for GST in insects.[12] The detoxification function of these enzymes has a particular signifi-

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cance in insects because it contributes to the development of resistance to insecticides by catalyzing their degradation.[14,15] GST have been the last class of detoxification enzymes recognized as important in insecticide resistance. Nevertheless they play a primary role in resistance to some organophosphorus insecticides and probably a secondary role in resistance to other insecticides such as gamma-hexachlorocyclohexane.[16] The toxicity of a number of insecticides is synergized by inhibitors that block oxidative metabolism.[17] Many organophosphorus and chlorinated hydrocarbon insecticides are metabolized in insects by reactions involving GST enzymes.[18,19]

GST are very susceptible to inhibition by a variety of compounds.[13] Miyamoto and Yamamoto[20] described a weak inhibition of housefly GST by chlorophenylchalcones but a strong inhibition by the glutathione (GSH) conjugates of those chalcones. Hodnick *et al*[21] found that chronic ingestion of quercetin resulted in depressed activities of GST, glutathione reductase, glutathione peroxidase, and also peroxidase and catalase, suggesting that quercetin partially inhibits the insect antioxidant enzyme system.

Insecticide activity synergized by inhibitors of GST in insects has been reported,[13,20,22] but none has been reported in Hemiptera (Reduviidae), especially in vectors of Chagas disease.

In the search for selective insecticide synergists for chemical control of Triatominae, we have studied the distribution and properties of GST from *T. infestans*,[23] and also found natural products of the flavonoid type such as gossypol and quercetin and some triphenylmethane dyes (thymol blue) capable of inhibiting *T. infestans* GST *in vitro*.[24]

In the present paper, we report an approach to increase the insecticidal activity of an insecticide, fenitrothion, against *T. infestans*, by using co-treatment with sub-lethal doses of quercetin or thymol blue and we have tried to reveal the role of GST in this type of synergism.

2 MATERIALS AND METHODS

2.1 Chemicals

1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from Janssen Chemical, Beerse Belgium. [*ring-methyl*-^{14}C]fenitrothion, specific radioactivity 141 mCi g^{-1}, was a gift from Sumitomo Chemical Co. Japan. Desmethyl-fenitrothion was synthesized according to Eto *et al*.[25] All other reagents were purchased from Sigma Chemical Co. USA.

2.2 Biological material

T. infestans were obtained from a colony maintained in our laboratory at 30°C and 50–60% RH over a period of 25 years.[26] The experimental work was done on *T. infestans* males 12–14 days old (160–180 mg body weight) starved for two weeks, and on nymphs II, one week old, starved since moulting.

2.3 Enzyme preparation

Adult *T. infestans*, starved for 30 days, were dissected, discarding guts and heads. They were then homogenized in distilled water, centrifuged at 10 000*g* for 10 min, and this first supernatant was filtered through glass wool and recentrifuged at 100 000*g* for 1 h. The supernatant so obtained was the source of GST activity and non-specific esterase activity. The first supernatant was used for acetylcholinesterase activity. Purified rabbit liver GST was purchased from Sigma Chemical Co. (USA), specific activity 150 units per mg of protein, assayed with 1.0 mM CDNB and 2.5 mM GSH.

2.4 Protein concentration

Protein concentrations in the homogenates were determined by the method of Lowry *et al*.[27] Bovine serum albumin was used as a standard.

2.5 Determination of enzyme activities

GST activity was assayed using the procedure described by Habig *et al*[28] using CDNB as substrate, in UV semi-microcuvettes (4 ml; Fisher Scientific), by sequential addition of phosphate buffer (pH 6.5; 0.1 M; 1.78 ml), enzyme preparation as above (0.1 ml), GSH solution in buffer (50 mM; 0.1 ml) and CDNB solution in acetonitrile (50 mM; 0.02 ml) (2.0 ml final volume of the routine incubation mixture). Enzyme activity was determined by continuously monitoring the change in absorbance at 340 nm for 3 min at 25°C with a Shimadzu UV-160 spectrophotometer.

Acetylcholinesterase (AChE) activity in *T. infestans* homogenates was assayed according to the procedure described by Wood *et al*[17] using acetylthiocholine as substrate and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) as chromogenic reagent. Non-specific esterase (NSE) activity in *T. infestans* homogenates was assayed according to the procedure described by Casabé *et al*[29] using phenylthioacetate as substrate and DTNB as chromogenic reagent.

2.6 Inhibition studies

The GST inhibitory potency of quercetin or thymol blue was ascertained by incubating *T. infestans* homogenates with different concentrations of these compounds. The results were expressed as I_{50} (M), the inhibitor concentration to yield 50% of the non-inhibited GST activity (using CDNB-GSH as substrates). The I_{50} values or the pI_{50} values (negative logarithm of I_{50} values) were estimated graphically from three to five determinations around this point (in three replicates).

2.7 Toxicity testing

Adults of *T. infestans*, 12–14 days old, 160–180 mg body weight, starved for two weeks were used in toxicity tests. Fenitrothion solutions in acetone and GST inhibitors were applied topically to the tip of the insect's abdomen. The synergist candidates, quercetin and thymol blue, were applied 2 h before

fenitrothion. Quercetin solutions were prepared in acetone + 10% glycerol, and thymol blue solutions were prepared in acetone + ethanol (1 + 1 by volume) + 10% glycerol. Triplicates of 10 insects each were used for every treatment. At least three concentrations giving between 0% and 100% mortality were assayed. The topically treated insects were kept at 30°C and 50–60% RH. After 24 h treatment, mortality was assessed. The mortality data were analyzed with a computer program based on the probit method.[30] The synergistic effect was evaluated by the synergistic ratio (SR) calculated as: $SR = LD_{50}$ (fenitrothion)/LD_{50} (fenitrothion + quercetin or thymol blue).

2.8 In-vivo enzyme assay

Groups of five adult males were topically treated with quercetin (17 µg per insect) or thymol blue (120 µg per insect), before the topical application of fenitrothion (0.3 µg per insect). Treated insects were killed 4 h later, homogenates prepared as described, and GST, AChE and NSE activities were assayed as described above.

2.9 Thin layer chromatographic analysis of [^{14}C]fenitrothion metabolism due to GST (*in vitro*)

T. infestans homogenates and commercial rabbit liver GST were separately incubated with [^{14}C]fenitrothion in order to study its GSH-dependent degradation alone and in the presence of 0.3 mM of quercetin (40 times the I_{50}) for CDNB-GSH assay. The micro-incubation system consisted of enzyme preparation (0.1 ml) in 0.02 M phosphate buffer pH 7.2 (total volume of 0.16 ml). [^{14}C]Fenitrothion was introduced in acetone solution (10 µl; 12 600 dpm) and GSH was added at a final concentration of 2.5 mM (50 µl). After 1 h incubation at 30°C the micro-incubation system was analyzed by TLC. The total incubation volume was spotted on TLC silica-gel plates (20 × 20 E. Merck 60 F_{245}) and an authentic non-radioactive standard of desmethyl-fenitrothion spotted onto a separate lane. The developing solvent was butanol + acetic acid + water (4 + 1 + 2 by volume). In the inhibition assays the quercetin was introduced in acetone (5 µl) and pre-incubated for 15 min.

The visualization of TLC plates was performed by radio-autography and the identification of radioactive spots by comparing directly with the non-radioactive standard (desmethyl-fenitrothion). The quantification of the metabolites was done by scraping off the spots from the TLC plates and counting them in Aquasol scintillation cocktail in a Beckman LS-7000 liquid scintillation counter.

2.10 [^{14}C]Fenitrothion toxicokinetics in *Triatoma infestans* adults with and without quercetin treatment

One group of seven *T. infestans* males were topically treated with acetone solution of [^{14}C]fenitrothion (10 µl; 70 000 dpm each), and another group of six males was topically treated with quercetin (17 µg each), immediately before the [^{14}C]fenitrothion (70 000 dpm).

2.10.1 Determination of non-penetrated [^{14}C]fenitrothion (wash-off technique)

Each insect was kept individually in an all-glass liquid scintillation vial sealed with perforated Parafilm. After 24 h, the insects were rinsed for 5–10 s in acetone (3 × 1 ml). The acetone rinses from each replicate were evaporated separately under a stream of nitrogen and the samples subjected to [^{14}C] quantitation by LSC (liquid scintillation counting); these values represented the external body rinse.

2.10.2 Determination of [^{14}C]excreted

Each vial that held treated insects and contained the insect faeces of 24 h was extracted in Aquasol (Liquid scintillation cocktail) and counted.

2.10.3 Determination of penetrated radioactivity

Insect heads were homogenized separately in liquid scintillation cocktail. Insect bodies were first extracted with ether and then re-extracted with methanol. The fractions were evaporated separately. Remaining carcasses were ground and homogenized in liquid scintillation cocktail. All fractions were subjected to [^{14}C] quantitation by LSC, obtaining the corresponding radiocarbon content for external body rinse, head, internal body (ether and methanol fractions), carcasses and excrements. The total radiocarbon recovery was 91–98% of the original dose.

2.11 In-vivo TLC-analysis of [^{14}C]fenitrothion metabolism in combined mixture of ether and methanol extracts from *Triatoma infestans* bodies treated with and without quercetin

Three males of *T. infestans* were topically treated with [^{14}C]fenitrothion (70 000 dpm) each and another three males were treated with quercetin (17 µg) and then with [^{14}C]fenitrothion each.

The insects were kept individually in vials, and after 24 h they were rinsed thoroughly with acetone, heads discarded and bodies homogenized, first with ether (3 × 3 ml) and then with methanol (3 × 3 ml). The combined ether and methanol extracts were evaporated under a stream of nitrogen and the reduced volumes spotted on TLC plates for chromatographic analysis. The chromatographic analysis was performed as described in Section 2.9. Total radiocarbon recovery was 83–89%.

3 RESULTS

3.1 Effect of quercetin or thymol blue on fenitrothion insecticide activity against *Triatoma infestans*

Using the LD_{50} values obtained in this study (Table 1) the synergistic ratio (SR) was calculated for quercetin and for thymol blue. The toxicity of the mixtures was significantly higher than the toxicity of

Table 1. Comparative topical toxicities of fenitrothion, fenitrothion + quercetin and fenitrothion + thymol blue in adult males of *Triatoma infestans*

	LD_{50} (*μg per insect*) (*confidence limits*)[ab]	*SR*[c]
Fenitrothion	0.775 (0.683–0.880)	1
Fenitrothion + quercetin[d]	0.411* (0.359–0.472)	1.89
Fenitrothion + thymol blue[e]	0.292* (0.222–0.385)	2.65

[a] Lichfield & Wilcoxon (Reference 30).
[b] * Significant difference from single fenitrothion treatment ($P < 0.05$; based on non-overlapping 95% confidence limits).
[c] Synergistic ratio (see Section 2.7).
[d] 17 μg per insect.
[e] 120 μg per insect.

fenitrothion alone ($P < 0.05$, based on non-overlapping 95% confidence limits).

3.2 In-vitro inhibition of *Triatoma infestans* glutathione *S*-transferase

The spectrophotometric assay for the GST of crude homogenates from *T. infestans*, using GSH and CDNB as substrates, demonstrated that the inhibition ability, measured as pI_{50} (Table 2) was higher for quercetin ($pI_{50} = 6.1$) than for thymol blue ($pI_{50} = 5.1$).

3.3 Glutathione *S*-transferase activity on [^{14}C]fenitrothion with and without inhibitors

The in-vitro incubation of *T. infestans* GST with [^{14}C]fenitrothion and GSH produced desmethyl-fenitrothion as the main metabolite. The same result was obtained when the purified rabbit liver GST was used (Fig 1). The inhibition potency of quercetin on *T. infestans* GST activity towards [^{14}C]fenitrothion was in broad agreement with the results obtained using CDNB as the electrophilic substrate (Table 2). Three independent experiments showed a range of

Table 2. In-vitro inhibition of *Triatoma infestans* glutathione *S*-transferase activity by quercetin and thymol blue

Quercetin		*Thymol blue*	
pI[a]	*Inhibition*[b] (% (± *SD*))	*pI*[a]	*Inhibition*[b] (% (± *SD*))
7.0	13 (±1)	6.3	4 (±1)
6.3	40 (±3)	6.0	16 (±1)
6.1	50 (±1)	5.3	37 (±2)
6.0	53 (±3)	5.0	53 (±2)

[a] pI: negative logarithm of molar concentration of inhibitor, Substrate: CDNB.
[b] Data are the average of four independent experiments.

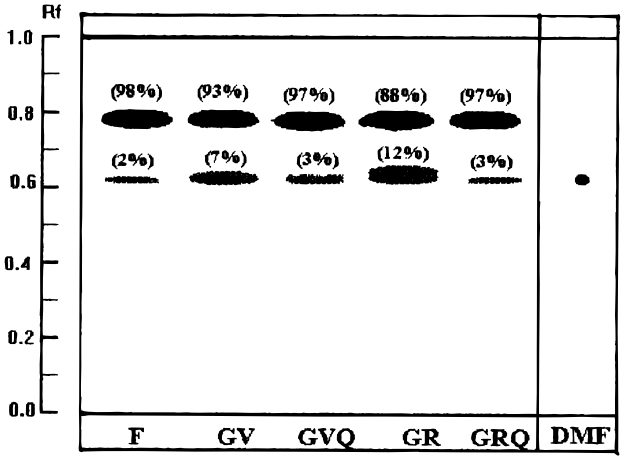

Figure 1. Representative autoradiogram of thin-layer chromatographic separation of in-vitro incubation of [^{14}C]fenitrothion with GST as indicated (See Section 2.9 for experimental details). Lane F is parent [^{14}C]fenitrothion (12 600 dpm) in 0.02 M phosphate buffer pH 7.2 (1 h; 30°C). Lane GV corresponds to *T. infestans* GST + GSH + [^{14}C]fenitrothion. Lane GVQ corresponds to *T. infestants* GST + GSH + quercetin (3×10^{-4} M) + [^{14}C]fenitrothion. Lane GR corresponds to rabbit liver GST + GSH + [^{14}C]fenitrothion. Lane GRQ corresponds to rabbit liver GST + GSH + quercetin (3×10^{-4} M) + [^{14}C]fenitrothion. Lane DMF corresponds to authentic desmethylfenitrothion. The dried TLC plate was exposed to X-ray film for 12 days. The radioactivity recovery was 88 (± 6)% (three independent experiments).
The samples were spotted at O (origin);
R_f: 0.8 = unmetabolized [^{14}C]fenitrothion;
R_f: 0.60 = desmethyl-[^{14}C]fenitrothion. Relative radioactivity percentage (LSC) of spots is indicated in brackets.

1–3% of desmethyl-[^{14}C]fenitrothion as a background due to non-enzymatic degradation. The in-vitro incubation of [^{14}C]fenitrothion with male *T. infestans* homogenates (1 h, 30°C) gave desmethyl-[^{14}C]fenitrothion as an important metabolite (5% of total radioactivity, discounting the background). The same principal metabolite was obtained when commercial purified rabbit liver GST was incubated with [^{14}C]fenitrothion under the same conditions (10% of total radioactivity, discounting the background). When either of these enzymes was incubated with [^{14}C]fenitrothion in the presence of 3×10^{-4} M quercetin, the metabolite concentration diminished to the background level (Fig 1).

3.4 In-vivo enzyme levels following single and combined doses of fenitrothion with and without GST inhibitors

The synergistic topical dose of quercetin in *T. infestans* males gave a pattern of acetylcholinesterase (AChE) and non-specific esterases (NSE) similar to the control but a significantly lower ($P < 0.01$, *t*-test) GST activity (57% inhibition) (Table 3). Fenitrothion single doses showed a pattern of GST activity similar to the control, but AChE and NSE levels were significantly lower than the control (25% inhibition, $P < 0.05$, and 40% inhibition, $P < 0.01$, respectively). The combined topical dose of fenitrothion plus quercetin showed a GST activity pattern

Assays	Specific enzyme activity[a] (μmol min^{-1} mg^{-1} protein) (±SD)		
	Glutathione S-transferase	Acetylcholin esterase	Non-specific esterase
Control	35 (±5)	0.036 (±0.004)	30 (±4)
Fenitrothion[b]	34 (±5)	0.027 (±0.002)[f]	18 (±2)[e]
Quercetin[c]	15 (±1)[e]	0.034 (±0.002)	30 (±3)
Fenitrothion + quercetin	15 (±1)[e]	0.015 (±0.001)[e]	18 (±2)[e]
Thymol blue[d]	17 (±2)[e]	0.038 (±0.002)	30 (±4)
Fenitrothion + thymol blue	19 (±3)[e]	0.014 (±0.002)[e]	24 (±4)

Table 3. In-vivo enzyme assays of male *Triatoma infestans* in fenitrothion, fenitrothion + quercetin or fenitrothion + thymol blue topically dosed insects

[a] Measured 4 h after treatment; $n = 3$.
[b] 0.3 μg per insect.
[c] 17 μg per insect.
[d] 120 μg per insect.
[e] Significant difference from control ($P < 0.01$; *t*-test).
[f] Significant difference from control ($P < 0.05$; *t*-test).

similar to the single quercetin dose, and the lowest level of AChE activity (59% inhibition, $P < 0.01$) and the same degree of NSE inhibition as in the single fenitrothion treatment (Table 3). When thymol blue was assayed in a similar way, the results showed 52% inhibition of GST activity ($P < 0.01$) and no inhibition of AChE or NSE. The combined dose of thymol blue and fenitrothion gave a similar low GST activity (45% inhibition, $P < 0.01$) and an even lower AChE activity (61% inhibition, $P < 0.01$) (Table 3).

The ingestion tests with thymol blue in nymphs II and males (*T. infestans*) showed no inhibition of AChE and NSE but a significant inhibition ($P < 0.01$) of GST in a 4-h treatment. Table 4 shows that nymph II GST was 38% inhibited and male GST was 70% inhibited compared to control levels.

These results demonstrated that thymol blue potency in inhibiting GST from *T. infestans* occurs not only in adults but also in nymphs, and was also evident in oral or contact exposures (Tables 3 and 4).

The in-vivo GST inhibition ability of quercetin and thymol blue was also shown by injection (Table 5), acting much more rapidly than in ingestion or contact tests. Four hours after injection of quercetin or thymol blue, GST activity in *T. infestans* males showed significant inhibition of 43% and 29%, respectively ($P < 0.01$). Quercetin and thymol blue injected in *T. infestans* males resulted not only in GST inhibition but also in enhancing the mortality ratio due to contact doses of fenitrothion (Table 5).

3.5 In-vivo metabolism of [^{14}C]fenitrothion topically applied to *Triatoma infestans* males with and without quercetin

Cuticular penetration of [^{14}C]fenitrothion was similar in both groups, with only 10% (6–12% range) of the topically applied insecticide remaining on the surface after 24 h (external rinse, Table 6). The internal radiocarbon content of heads was also similar in both treatments (1% of the total recovered radiocarbon). Within the carcasses, 8–21% of the

Enzyme assayed[a]		Specific enzyme activity (μmol min^{-1} mg^{-1} protein) (±SD)[ab]	
		Control[d]	Thymol blue[e]
Glutathione S-transferase	Nymph II	16 (±1)	10 (±1)*
	Male	35 (±2)	11 (±1)*
Acetyl cholinesterase	Nymph II	0.027 (±0.002)	0.026 (±0.002)
	Male	0.036 (±0.002)	0.038 (±0.002)
Non-specific esterases	Nymph II	28 (±2)	27 (±2)
	Male	30 (±2)	25 (±2)

Table 4. In-vivo enzyme activities in nymphs II and males of *Triatoma infestans*, after oral feeding with thymol blue

[a] Measured 4 h after treatment.
[b] * Significant difference from control ($P < 0.01$; *t*-test).
[c] Data represent the average of two independent experiments.
[d] Artificial feeding with NaCl 0.15 M solution (1 h) (Reference 31).
[e] Artificial feeding with thymol blue 10^{-3} M solution in NaCl 0.15 M (1 h). Oral dose calculated in male: 44 μg per insect. Oral dose calculated in nymph II: 18 μg per insect.

Treatment[a]	Glutathione S-transferase activity ($\mu mol\ min^{-1}\ mg^{-1}$ protein)[b,c] ($\pm SD$)	24-h% Mortality (%)
Control[d]	35 ($\pm$ 2)	0
Quercetin[e]	20 ($\pm$2)*	0
Thymol blue[f]	25 ($\pm$2)*	0
Fenitrothion[g]	36 ($\pm$2)	20
Quercetin + fenitrothion[g]	21 ($\pm$2)*	100
Thymol blue + fenitrothion[g]	27 ($\pm$2)*	100

[a] Injections were performed following Picollo *et al* (Reference 32).
[b] Enzyme activity measured 4 h after treatment (CDNB-GSH assay).
[c] * Significant difference from control ($P < 0.01$; *t*-test).
[d] Injection of 2 µl of distilled water per insect.
[e] Injection of 2 µl of 10^{-2} M quercetin per insect.
[f] Injection of 2 µl of 10^{-2} M thymol blue per insect.
[g] Topical application of 10 µl0.1 mg ml^{-1} solution in acetone.
[h] Topical application of fenitrothion 2 h after injection of quercetin or thymol blue.

Table 5. Effect on GST and mortality of injected doses of quercetin and thymol blue in males of *Triatoma infestans*

Fraction analyzed		Recovered radiocarbon (% of total)[a,b]	
		[^{14}C]fenitrothion[c]	Quercetin + [^{14}C]fenitrothion[d]
External body rinse		10 ($\pm$4)	11 ($\pm$1)
Head		1 ($\pm$0.2)	1 ($\pm$0.2)
Internal body content	Ether	38 ($\pm$1)	42 ($\pm$3)
	Methanol	31 ($\pm$4)*	23 ($\pm$3)*
Carcasses		12 ($\pm$4)	17 ($\pm$4)
Excrement		8 ($\pm$3)	6 ($\pm$1)
Total		(100)	(100)

[a] Radiocarbon recovery: 91–98%.
[b] * Values differ significantly ($P < 0.05$; *t*-test).
[c] Data are the average of seven insects treated independently. [^{14}C]fenitrothion: 70 000 dpm per insect.
[d] Data are the average of six insects treated independently. Quercetin: 17 µg per insect.

Table 6. Recovered radiocarbon percentage in fractions of male *Triatoma infestans* 24 h after contact administration of [^{14}C]fenitrothion or quercetin + [^{14}C]fenitrothion

Compound	R_f	Recovered radioactivity[a,b] (% of total) ($\pm SD$)	
		[^{14}C]fenitrothion[c]	Quercetin + [^{14}C]fenitrothion
Fenitrothhion	0.76	18 ($\pm$4)	35 ($\pm$4)*
Desmethylfenitrothion	0.58	35 ($\pm$1)	30 ($\pm$1)*
Unknown	0.44	8 ($\pm$1)	7 ($\pm$4)
	0.38	6 ($\pm$1)	6 ($\pm$1)
	0.30	13 ($\pm$2)	6 ($\pm$2)
	0.26	11 ($\pm$2)	8 ($\pm$1)
	0.14	9 ($\pm$1)	8 ($\pm$1)
Total	—	100	100

[a] Data represent the average of three independent experiments. Radiocarbon recovery: 83–89%.
[b] * Significant differences from single fenitrothion treatment ($P < 0.01$; *t*-test)

Table 7. Recovery of metabolites from combined ether and methanol extracts of *Triatoma infestans* separated by thin-layer chromatography after single topical application of [^{14}C]fenitrothion or combined does of quercetin + [^{14}C]fenitrothion

total recovered radiocarbon was present as non-extractable radioactivity, with average values of 12% and 17% for fenitrothion and combined quercetin plus fenitrothion treatments respectively. The recovered radiocarbon content measured in excrement ranged from 5 to 11%, with average values of 8% and 6%, respectively. None of these results showed statistically significant differences between the two treatments ($P > 0.05$, t-test) (Table 6). Concerning internal radioactivity, the level of polar metabolites (methanol extracts) was significantly lower in the combined treatment than in the single [^{14}C]fenitrothion-dosed insects ($P < 0.05$, t-test) (Table 6). On the other hand, the internal radioactivity extracted by ether, representing mainly parent compound residues and non-polar metabolites, was similar in both treatments, ranging from 37 to 45% with averages of 38 and 42% ($P > 0.05$, t-test) (Table 6).

3.6 Metabolite analysis and chromatography

Insects dosed with quercetin plus [^{14}C]fenitrothion showed a higher level ($P < 0.01$) of the parent compound (unmetabolized [^{14}C]fenitrothion) and a correspondingly lower level ($P < 0.01$) of desmethyl-[^{14}C]fenitrothion, than those treated only with [^{14}C]fenitrothion (Table 7).

In a separate experiment, insects topically treated with fenitrothion plus piperonyl butoxide yielded partial inhibition of the unknown metabolites (R_f = 0.44–0.14), suggesting a mixed-function oxidase contribution. Thus, the in-vivo chromatographic analysis and the quantitation of the different metabolites showed that desmethyl-fenitrothion was the main degradation product due to GST activity and it was significantly diminished in insects co-treated with quercetin.

4 DISCUSSION

This report represents the first study to demonstrate that GST inhibition could be the mechanism responsible for the synergism of a dimethyl-phosphorothionate in Chagas' disease vectors. Thus, fenitrothion insecticide activity against *T. infestans* has been significantly increased by co-treatment of the bugs with sub-lethal doses of quercetin or thymol blue. Our data demonstrate clearly that the toxicity of mixtures such as quercetin plus fenitrothion or thymol blue plus fenitrothion is significantly higher than that of fenitrothion alone. In a previous study[23] we demonstrated that the GST from *T. infestans* is able to generate aqueous metabolites when incubated *in vitro* with malathion, parathion or fenitrothion. This *T. infestans* soluble enzyme is present in every developmental stage and widely distributed in all insect organs. After purification (6-fold), it gives a unique molecular form which is catalytically active using methyl iodide as substrate in polyacrylamide gel electrophoresis (PAGE). One of the most active substrates is CDNB and the kinetics are complex, resembling those reported for glutathione *S*-transferase A from rat liver, showing a bi-phasic kinetic mechanism in which the reaction pathway depends on the GSH concentration. In general, the properties of this insect enzyme are similar to those of enzymes isolated from vertebrate organisms.[23] In a subsequent study we have demonstrated the activity of some in-vitro inhibitors. Thus, fixed concentrations of natural products of flavonoid type, such as gossypol (0.1 mM) and quercetin (0.02 mM), were able to inhibit 66% and 78% of GST activity present in crude preparations of nymph V of *T. infestans*, respectively. In addition, we have found that triphenylmethane dyes such as phthaleins, fluoresceins and sulfophthaleins (0.1 mM) inhibited *T. infestans* GST activity in the range of 20–80%.[24]

In order to find the rationale for the fenitrothion synergism, we have quantified the anti-GST activity in this study, finding that quercetin is a more potent inhibitor *in vitro* than is thymol blue. Furthermore, we were able to demonstrate that treatments of insects with quercetin or thymol blue at sub-lethal synergistic doses were responsible for in-vivo GST inhibition. Thus, thymol blue topical, oral or injected treatments and quercetin topical or injected doses yielded significant inhibition of GST. Also evident was GST inhibition by thymol blue in topical or oral treatments, not only in *T. infestans* adults but also in nymphs II. Comparison of all three routes of administration showed that, in injection assays, thymol blue or quercetin acted much more rapidly than in ingestion or contact ones. In every assay, the co-treatment of quercetin or thymol blue plus fenitrothion produced not only inhibition of GST activity, but also a significantly enhanced inhibition of AChE that closely correlated with the lowering of GST activity. Quercetin or thymol blue injected or topically applied to *T. infestans* males resulted not only in GST inhibition, but also in enhancement of the mortality ratio due to fenitrothion treatment, which in turn presented a significantly inhibited pattern of AChE. This enhanced AChE inhibition was most likely due to the partial blockage of the GSH-GST detoxification route by quercetin or thymol blue in the OP-intoxicated insects. These results suggest that the synergistic topical dose of fenitrothion plus quercetin or fenitrothion plus thymol blue could be responsible for lowering the detoxification activity mediated by GST and enhancing AChE inhibition.

The available information on the observed rates of dealkylation indicates that GST will *O*-dealkylate both phosphorothionates and phosphates, and the dimethyl ester is a preferred substrate.[19]

The inhibitory potency of quercetin on the GST activity of *T. infestans* towards [^{14}C]fenitrothion was in accordance with the results obtained using CDNB as the electrophilic substrate. The in-vitro incubation of [^{14}C]fenitrothion plus GSH with male *T.*

infestans homogenates gave desmethyl-[^{14}C]fenitrothion as an important metabolite. The same main metabolite was obtained when commercially purified rabbit liver GST was incubated with [^{14}C]fenitrothion under the same conditions. The production of desmethyl-[^{14}C]fenitrothion *in vitro* was absolutely dependent on GSH as a cofactor, suggesting the participation of GST in its generation. When either of these enzymes was incubated with [^{14}C]fenitrothion in the presence of 0.3 mM quercetin, the metabolite concentration diminished to the background level, suggesting that the production of this main metabolite was due to GST, which at the same time is inhibited by quercetin.

The toxicokinetics of [*ring-methyl*-^{14}C] fenitrothion studied in two insect groups, one co-treated with quercetin and the other without, showed that the absorption, biotransformation and excretion performed by *T. infestans* produces significant differences only in the amount of polar fraction of internal body radioactivity. Actually the recovered polar radiocarbon expressed as a percentage of administered [^{14}C]fenitrothion in insects 24 h after combined treatment was lower than in insects dosed only with [^{14}C]fenitrothion. Thus, the combined contact exposure to quercetin plus [^{14}C]fenitrothion showed a decrease in the detoxification pathway compared to treatment with fenitrothion alone, and this is probably due to GST in *T. infestans*. Furthermore, the chromatographic analysis and the quantitation of the different metabolites showed that desmethyl-fenitrothion was the main degradation product of *T. infestans* GST activity and it was significantly diminished in insects co-treated with quercetin.

The experimental evidence obtained suggests that *T. infestans* is more susceptible to intoxication by fenitrothion when its GST detoxification pathway is totally or partially inhibited. The exposure to GST inhibitors like quercetin or thymol blue reduces the detoxification pathway of the organophosphorus compounds and thus leads to greater AChE inhibition. GST inhibition in *T. infestans* could be used as an initial model for the development of a new type of more effective triatomicidal formulation.

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